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ULTRASTRUCTURAL STUDIES OF EXPERIMENTAL VESICULATION. II. COLLAGENASE*

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Ultrastructural investigation of many of the natural blistering diseases (1–12) has provided valuable data from which hypotheses can be advanced concerning pathogenic mechanisms involved in the disease processes. Often, however, the study of natural blisters is difficult: the occurrence and development of natural vesicles cannot be controlled and interpretation of data gained from ultrastructural studies of such blisters is commonly complicated by the occurrence of several diverse simultaneous events such as inflammation and hemorrhage.

A useful adjunct to the study of the pathophysiology of the natural blistering diseases is the ultrastructural analysis of carefully controlled experimentally produced blisters. Using a variety of chemical agents, including proteolytic enzymes, we have attempted to produce experimental blisters which are ultrastructurally similar to those of certain natural blistering diseases or have some alterations in common with the natural diseases. Vesiculation produced by chemical agents may also reveal processes which, although not observed in natural disease, provide significant information about the structure and function of certain ultrastructural elements of the skin. For example, the response of the components of the dermal-epidermal junction and of desmosomes to a variety of chemical agents has theoretical importance since the chemistry of these structures is poorly understood and alterations in these structures are often associated with a variety of blistering and other pathologic processes.

In this paper we present our findings in blisters produced by Clostridial collagenase. This enzyme was chosen in the expectation of simulating blisters in which extensive damage to the upper dermal collagen has been observed as in epidermolysis bullosa dystrophica (4, 6). Surprisingly the most striking action of collagenase was observed to be directed, not against the dermal collagen fibrils, but against the basement membrane of the dermal-epidermal junction.

METHODS AND MATERIALS

Clostridial collagenase (Worthington Chemical Company) was dissolved in a phosphate buffer of pH 7.4 and injected intracutaneously into guinea pigs in concentrations of 0.04% to 2.0%. Punch biopsies were taken from one to twenty minutes after injection of the enzymes. For control studies, the pH 7.4 phosphate buffer was injected intracutaneously into guinea pigs and biopsies were taken at five and 20 minutes after injection. The tissue was fixed in either 2.0% osmium tetraoxide buffered with s-collidine (13) or 3.0% glutaraldehyde (14) with subsequent postfixation in 2.0% osmium tetraoxide and embedded in Epon (15). Following staining with methanolic uranyl acetate (16) and lead citrate (17) thin sections were examined with a Siemens Elmiskop I operating at 80 kv. Formalin fixed specimens were processed by routine methods for light microscopy.

After initial experiments had been carried out with a purified collagenase preparation a highly purified enzyme preparation became available. The experiments were repeated with the highly purified collagenase. No significant differences between the effects of the two collagenase preparations were detected except that gross and microscopic alterations were somewhat delayed in experiments using the highly purified agent.

RESULTS[1]

Blisters were observed to form rapidly following the intracutaneous injection of Clostridial collagenase. Grossly visible blisters were formed within one minute after injection of 0.04% collagenase. Dermal-epidermal separation was found in all blisters produced by collagenase but there was no evidence of

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[1] A micrograph of the normal dermal-epidermal junction in the guinea pig is included for comparison with the junctional changes induced by collagenase (Fig. 1). A brief but more complete description of the junction was presented in an earlier paper (12).

acantholysis or other forms of disruption of the normal epidermal cellular organization.

In the control studies, where phosphate buffer of pH 7.4 was injected intracutaneously into guinea pigs, blistering was not observed. Examination by both light and electron microscopy did not reveal dermal-epidermal separation.

Light microscopic observations demonstrated that blisters produced by all concentrations of the enzyme were essentially similar. Clean dermal-epidermal separation had occurred and occasional perinuclear vacuoles were noted in the basal cells. The dermis was edematous but there was no apparent alteration of the collagen bundles. Infiltrates of erythrocytes and inflammatory cells were also seen in the dermis.

Electron microscopic examination of the collagenase induced blisters revealed consistent and specific alterations of the dermal-epidermal junction and particularly of the basement membrane. The roofs of the vesicles were formed by epidermis and fragments of basement membrane associated with the basal border, and the floors of the blisters consisted of the upper dermal margin along which segments of basement membrane were present (Figs. 2 and 3).

Fragments of the basement membrane associated with the basal cell border tended to be present at sites where hemidesmosomes were located and absent at regions of the basal border between hemidesmosomes (Fig. 4). Fragments of basement membrane associated with the upper dermal limit retained relatively normal positional relationships with the various dermal elements.

In addition to being fragmented in the collagenase produced blisters, the basement membrane also demonstrated loss of the normally present linear dimension in certain areas of damage. In such regions diffuse extension of the basement membrane was noted and was most evident at the blister origin (Fig. 5). In several blisters, apparent splitting of the basement membrane was noted at the blister origin so that basement membrane was seen to be present concurrently along both the basal cell margin and the upper dermal limit (Fig. 6).

Within the blister cavity, occasional membrane bound cytoplasmic fragments were observed and, in some blisters, diffuse granular debris was seen to fill the entire cavity. Occasionally, intact free melanocytes were present within the blister cavity as well as intact free nuclei. It is probable that the nuclei were originally present in melanocytes and were then extruded into the blister cavity (Figs. 7 and 8).

A broad spectrum of basal cell damage was noted, ranging from cells which appeared intact and normal to those which showed obvious evidence of damage (Fig. 9). The degree of basal cell damage did not correlate with enzyme concentration or time of exposure to the enzyme. The chief manifestations of basal cell injury consisted of extrusions of membranous processes into the blister cavity usually at regions where there was a defect in the basement membrane (Fig. 2). Other features of damage included perinuclear vacuolization, mitochondrial swelling, and occasional fragmentation of the basal cell plasma membrane. Focal cytoplasmic degradation (18) was not observed and cells above the basal layer did not show evidence of injury.

In most blisters the dermis was edematous and the collagen fibrils appeared ultrastructurally normal. However, in blisters produced by exposure to 2.0% collagenase for ten minutes the blister cavity apparently formed with extensive injury to the upper dermis (Fig. 10). In the upper dermal zone large amounts of coarsely granular debris were observed as well as occasional membrane bound cytoplasmic fragments. Infrequent aggregates of collagen fibrils with their characteristic periodicity were seen to be closely associated with the accumulations of granular debris. In the blisters produced by 10 minute exposures to 2.0% collagenase, the basement membrane was identified along the basal cell border and showed evidence of injury (Fig. 11). The normally present "intermembranous space" was obliterated and the basement membrane itself no longer retained its characteristic linearity. In some regions of the blister, the basement membrane appeared to extend into the blister cavity and merge with the granular debris.

Immediately adjacent to the blister, occasional changes were observed along the dermal-

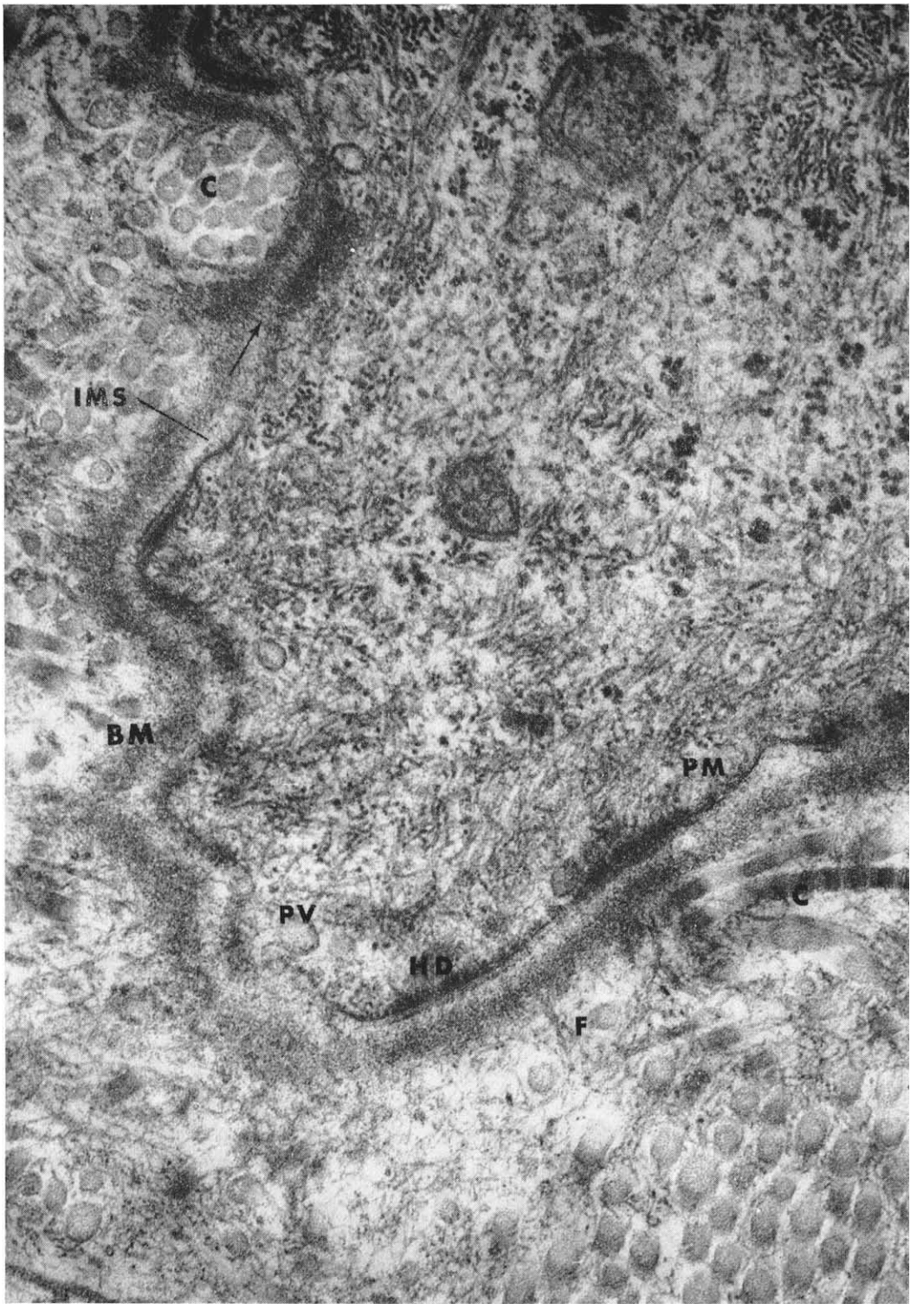

FIG. 1. Dermal-epidermal junction of normal guinea pig. Along the basal cell plasma membrane (PM) lie hemidesmosomes (HD) and pinocytotic vesicles (PV). The basement membrane (BM) parallels the basal cell plasma membrane from which it is separated by the "intermembranous space" (IMS) which is traversed at discrete points by fine filaments (arrow). Salient features of the basement membrane are moderate electron density, constant positional relationship to the basal cell plasma membrane and relatively constant linearity. The dermal elements include collagen fibrils (C) and numerous fine dermal filaments (F) some of which seem to insert into the basement membrane. Approximately 45,000 ×.

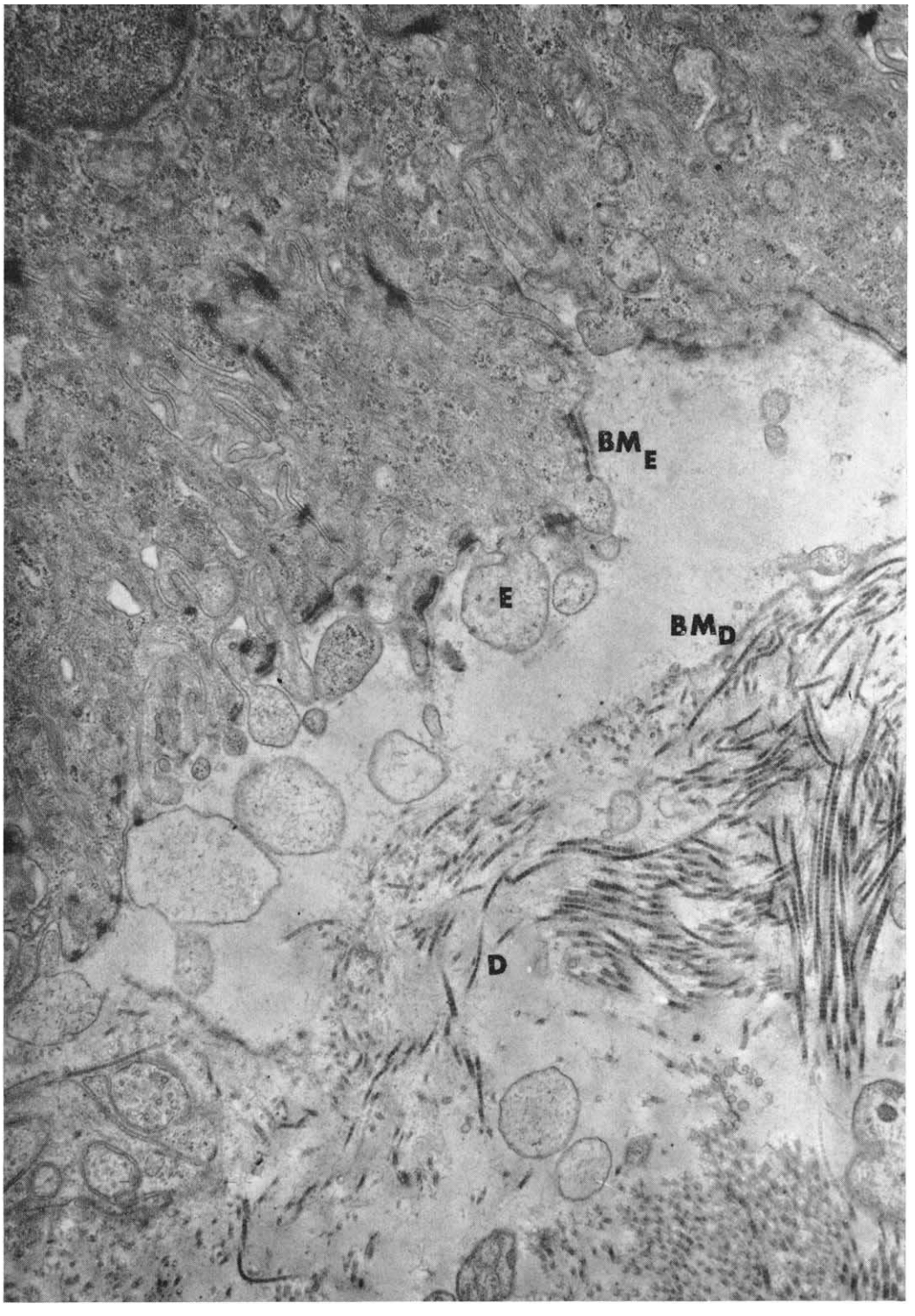

FIG. 2. 0.2% purified collagenase, one minute biopsy. Region of dermal-epidermal separation. Basement membrane fragmentation is apparent with portions of basement membrane associated with the basal cell margin (BM_E) and with the upper dermal limit (BM_D). Membranous extrusions of basal cells (E) extend into the blister cavity especially at points where there is a defect in the basement membrane. The dermis (D) is edematous but the collagen fibrils appear ultrastructurally normal. Approximately 10,000 ×.

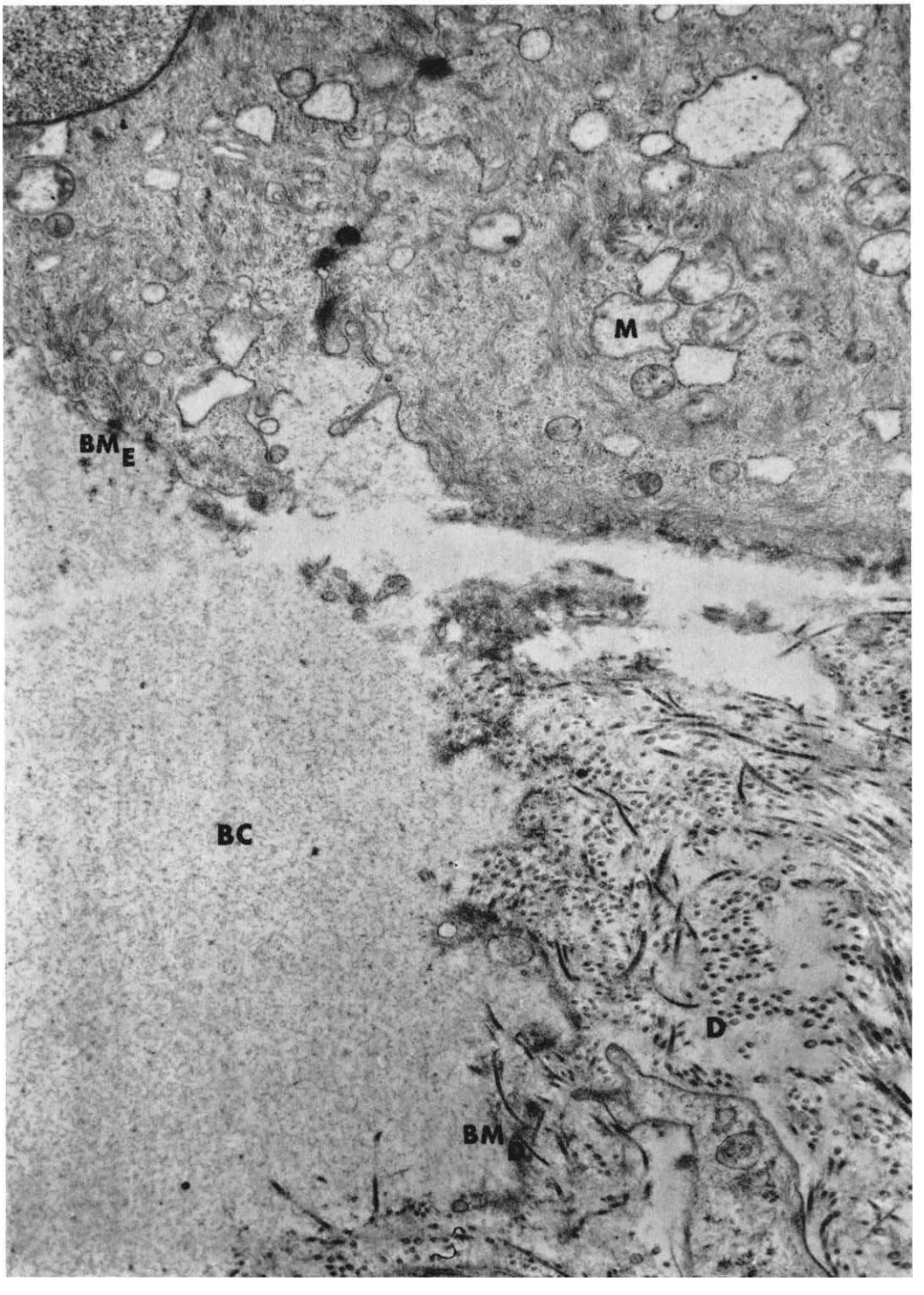

FIG. 3. 0.04% highly purified collagenase, twenty minute biopsy. Blister origin. Basement membrane is present along the basal cell border (BM_E) and along the upper dermal limit (BM_D) and is fragmented. The blister cavity (BC) is filled with finely granular debris and the mitochondria (M) of the basal cells are swollen. The dermis is edematous (D) and the collagen fibrils are unaltered. Approximately 7,500 ×.

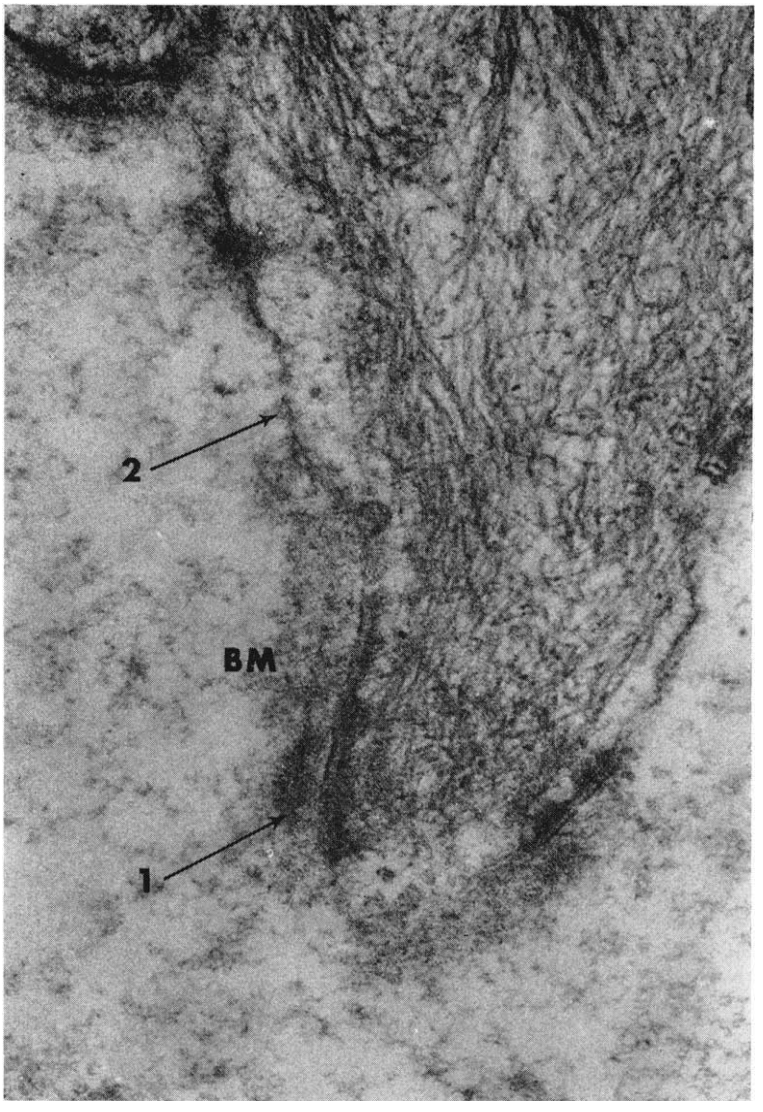

FIG. 4. 0.2% highly purified collagenase, six minute biopsy. Region of main blister. The disorganization of the basement membrane (BM) is manifested by fragmentation and obliteration of the "intermembranous space". Note that the basement membrane is present opposite hemidesmosomes (arrow 1) and absent opposite regions not occupied by hemidesmosomes (arrow 2). Approximately 60,000 ×.

epidermal junction in all collagenase induced lesions. These alterations consisted chiefly of pale swelling of the basal cell near the junction (Fig. 12). The basement membrane was closely applied to these pale extensions of basal cell cytoplasm but was more diffuse and indistinct than usual (Fig. 13). In the regions away from the blister the dermis was edematous and the collagen fibrils were ultrastructurally normal.

DISCUSSION

Enzymes capable of degrading native collagen at or near physiological pH have been designated as collagenases (19) and have been isolated from the filtrates of certain Clostridial cultures (20) as well as from cells cultured from the tail fins of metamorphosing anuran tadpoles (21, 22). A collagenase also has been isolated from colonies of *Streptomyces madurae* and *Trichophyton schoenleinii*

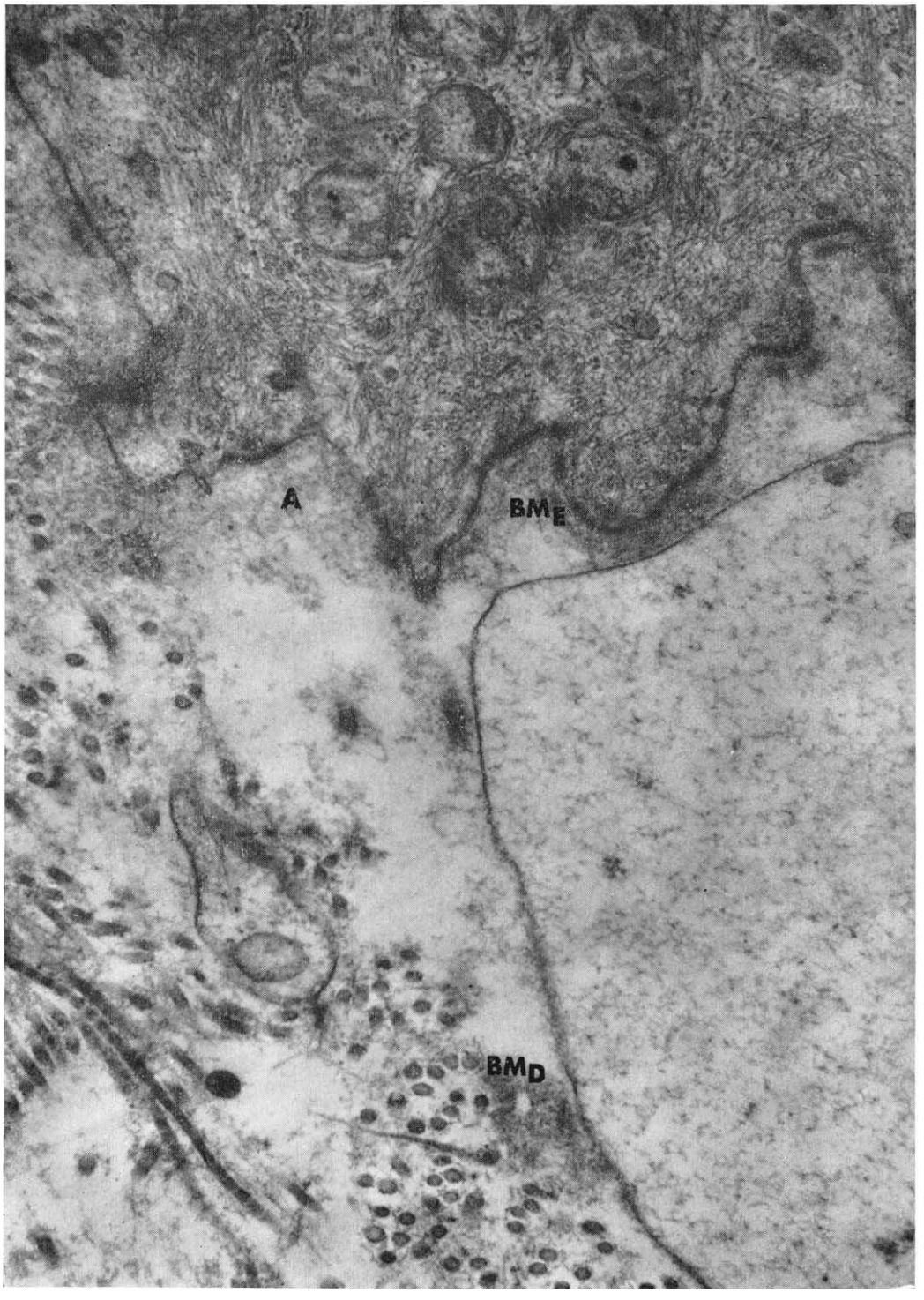

FIG. 5. 0.2% highly purified collagenase, six minute biopsy. Dermal-epidermal junction at blister origin. Injury to the basement membrane is evident with basement membrane extending along the basal cell border (BM_E) and partially along the dermal limit of the blister (BM_D). Attenuation of the basement membrane is indicated by the aggregation of diffuse finely granular material at the blister origin (A). Approximately 25,000 ×.

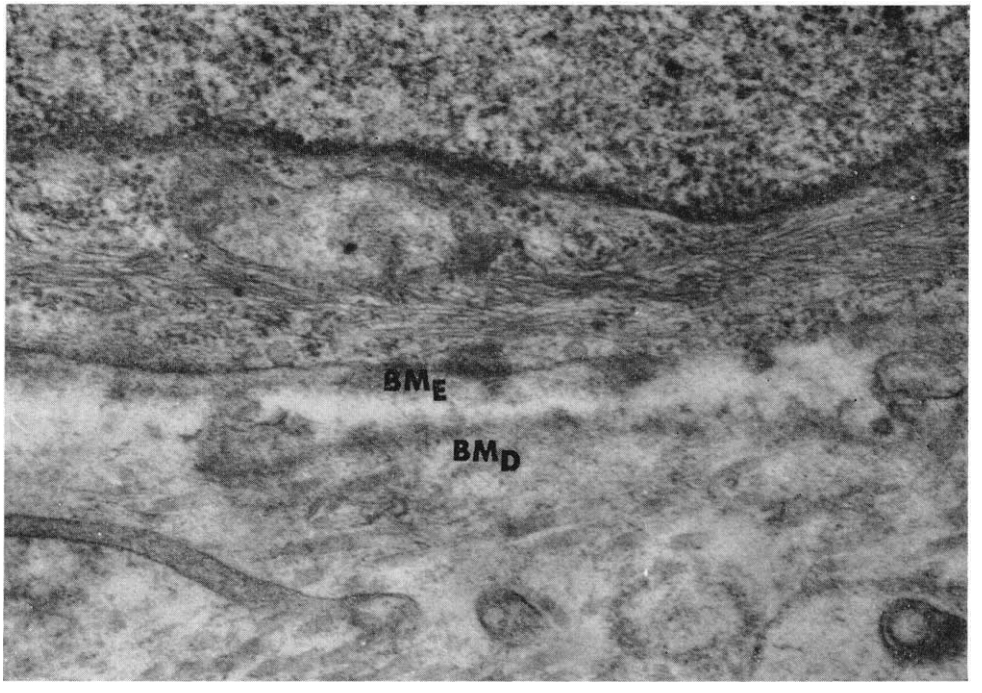

FIG. 6. 0.2% purified collagenase, one minute biopsy. Dermal-epidermal junction near blister origin. An apparent splitting of the basement membrane is seen with portions of basement membrane associated with the basal cells (BM_E) and with the dermis (BM_D). Approximately 55,000 ×.

(23). Data from several groups of investigators indicate that tadpole collagenase and Clostridial collagenase degrade collagen in different ways (21, 22, 24).

Techniques for the isolation of Clostridial collagenase of high purity have been developed (24–26) and purified Clostridial collagenase demonstrates a high degree of substrate specificity. In addition to degrading native collagen the enzyme acts also against denatured collagen, but has no effect on fibrin, keratin, casein or hemoglobin (25). Using synthetic peptides as substrates, it has been shown that Clostridial collagenase hydrolyzes specifically X-gly linkages producing N-terminal glycine (24, 27).

The action of Clostridial collagenase on skin has been studied by a number of investigators who, although using enzyme preparations of differing degrees of purity, reported essentially similar findings. Stoughton and Lorincz demonstrated that, following incubation of acetone-fixed sections of skin in a relatively impure collagenase preparation, there is a loss of Hotchkiss-McManus positive material from the basement membrane. Hambrick and Blank (29) reported dermal-epidermal separation in pieces of skin incubated in solutions of collagenase. And in addition to junctional separation, Einbinder et al (30) have observed injury to the collagen bundles after prolonged collagenase incubation.

Essentially, the results of these previous investigations of collagenase produced blisters, utilizing light microscopy and histochemistry, indicate that the enzyme initially produces clean dermal-epidermal separation perhaps by damaging the basement membrane as suggested by the work of Stoughton and Lorincz. The observations presented in this paper indicate that intracutaneously injected Clostridial collagenase also produces rapid and clean junctional separation and not a massive collagenolytic effect within the upper dermis except after prolonged exposure to high concentrations of the enzyme. Ultrastructural analysis of collagenase induced vesiculation reveals that there is a specific effect on the dermal-epidermal junction.

The ultrastructural disorganization of the

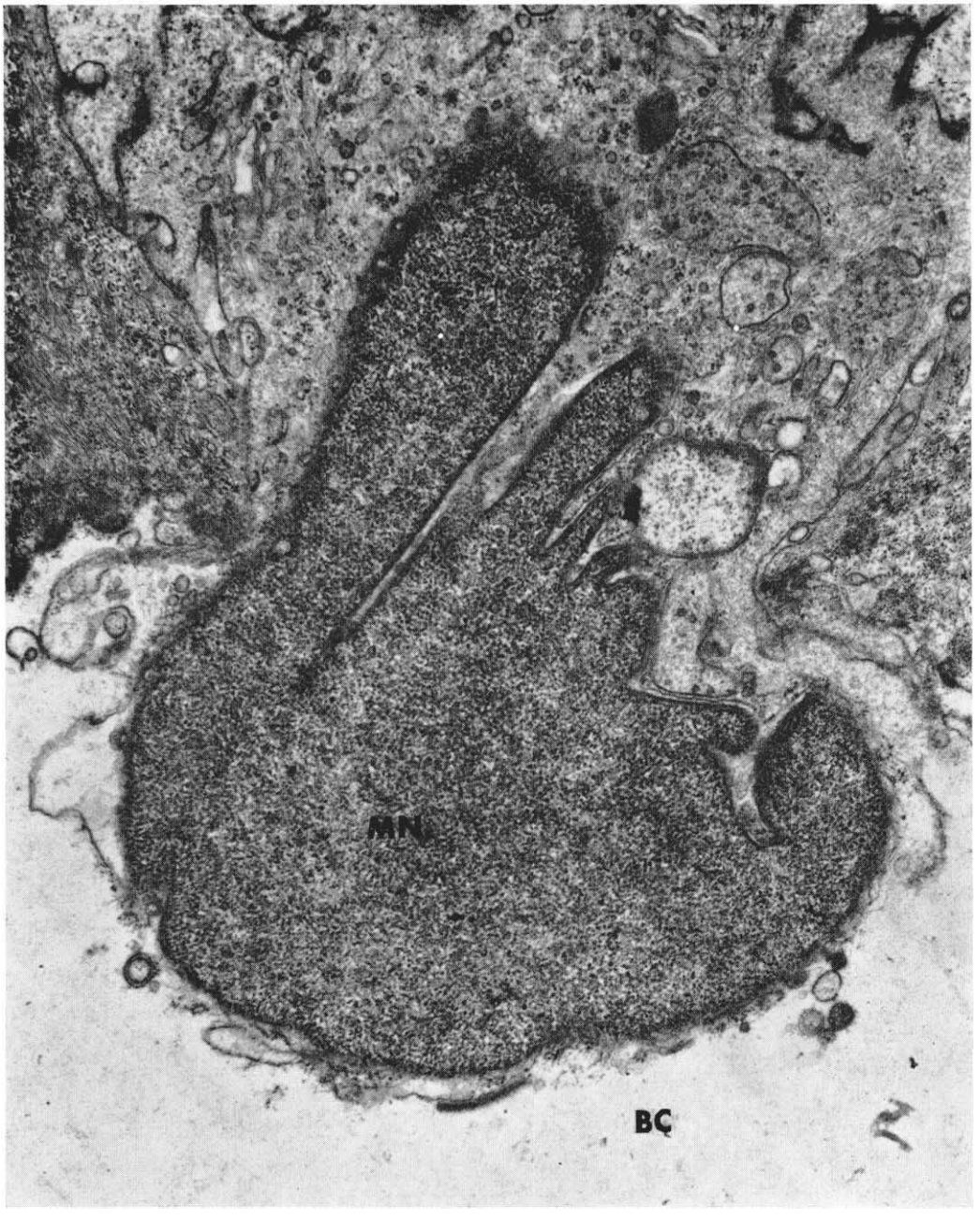

FIG. 7. 0.2% purified collagenase, one minute biopsy. Region of main blister. A melanocyte nucleus (MN) is being extruded into the blister cavity (BC). Approximately 12,500 ×.

basement membrane in collagenase blisters suggests that the enzyme is attacking this structure in a specific manner and that the basement membrane of the dermal-epidermal junction is composed of, in part, a collagen-like protein. Kefalides et al (31), investigating the chemical composition of isolated canine glomerular basement membrane, have reported that X-ray diffraction powder diagrams of their basement membrane preparation were similar to those of denatured collagen and pro-collagen. Also, they have dem-

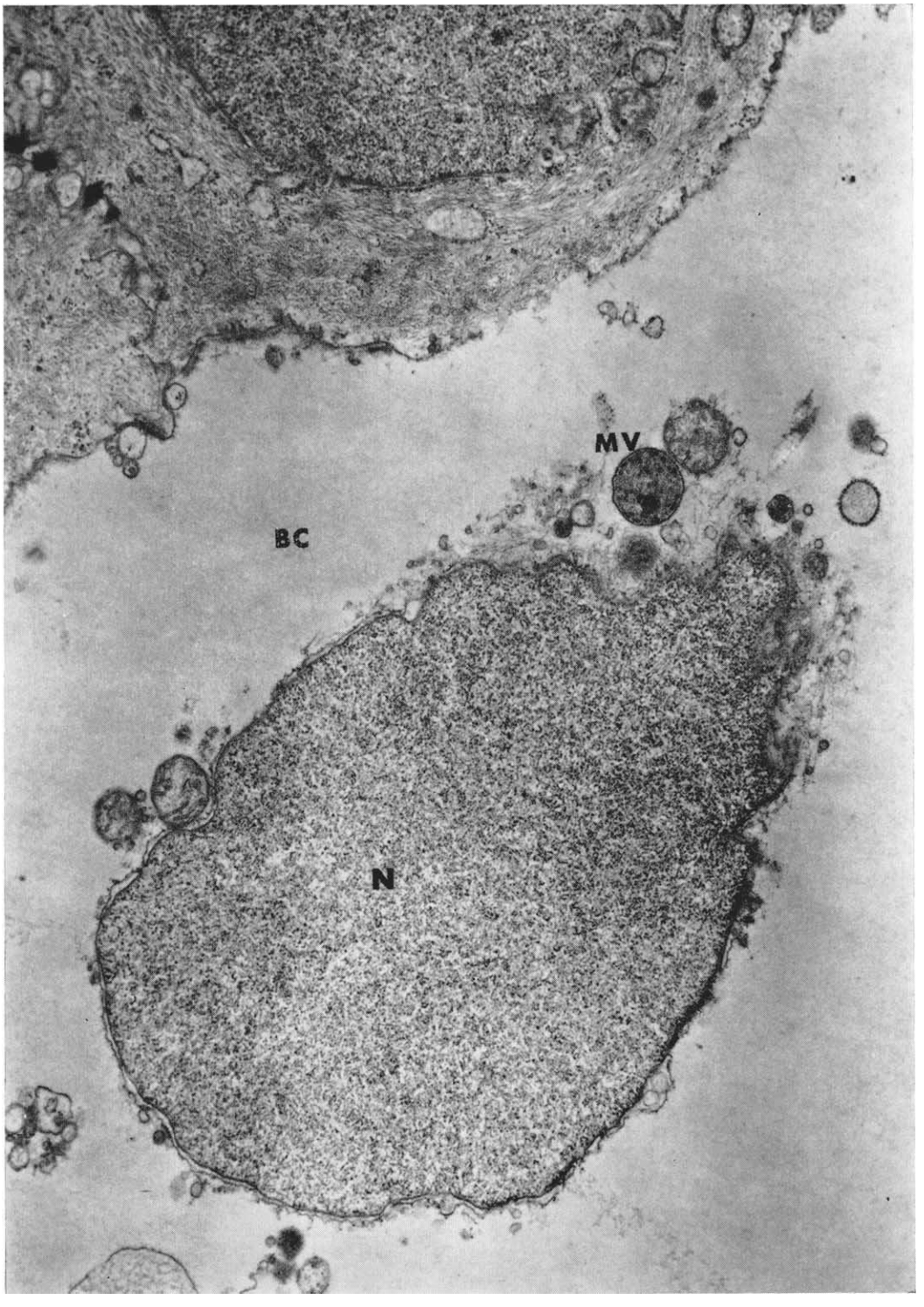

FIG. 8. 0.2% purified collagenase, one minute biopsy. Region of main blister. A nucleus (N) is free within the blister cavity (BC). Several organelles are associated with the nucleus including a multivesicular body (MV) suggesting that the nucleus is the remnant of a damaged melanocyte. Approximately 11,000 ×.

FIG. 9. 0.2% highly purified collagenase, six minute biopsy. Zone of major blister. The roof of the blister is composed of basal cells (BC) and fragments of basement membrane adhere to the basilar margin particularly at the hemidesmosomes (arrow). The two basal cells have large perinuclear vacuoles (V). Approximately 5,500 ×.

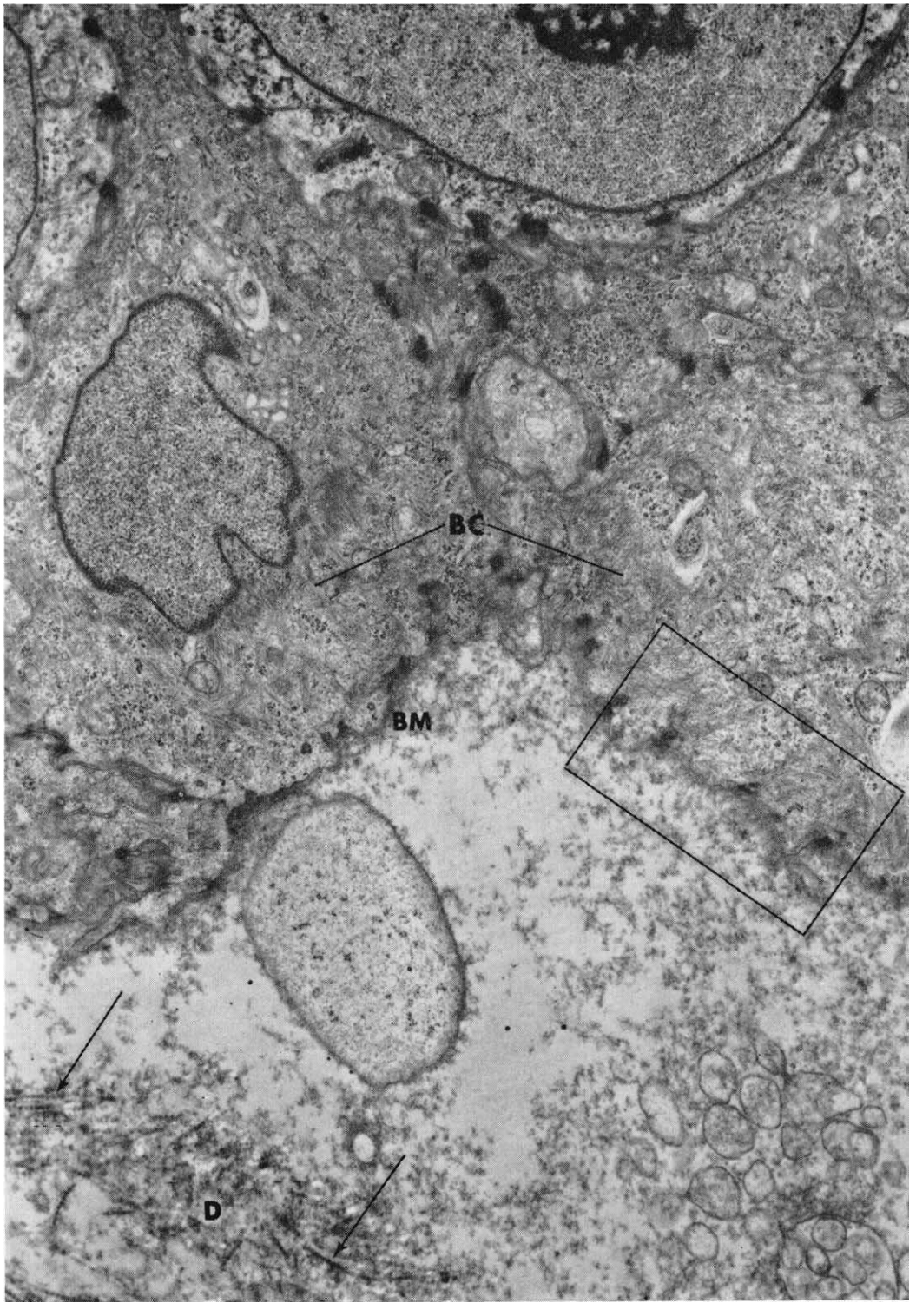

FIG. 10. 2.0% purified collagenase, ten minute biopsy. Dermal-epidermal junction at region of main blister. The cavity is composed of coarse granular debris (G) which merged with the extensively damaged dermis (D). Several recognizable collagen fibrils are associated with clumps of granular debris (arrow). The blister roof is composed of intact basal cells (BC) at the margin of which lies altered basement membrane (BM). Approximately 10,000 ×.

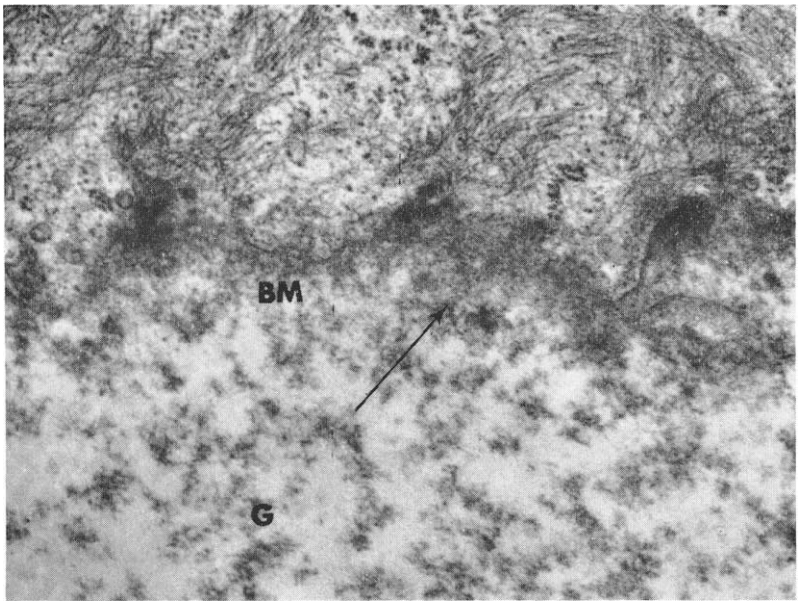

FIG. 11. 2.0% purified collagenase, ten minute biopsy. High magnification view of dermal-epidermal junction illustrated in Figure 10. The basement membrane (BM) is clearly altered. The "intermembranous space" is lost, clear linearity of the basement membrane is absent and the basement membrane appears to merge with the granular debris (G) of the blister cavity (arrow). Approximately 30,000 ×.

onstrated that canine glomerular basement membrane contains glycine, proline, and hydroxyproline in amounts less than have been reported for native collagen and hydroxylysine and cystein in amounts more than have been reported for native collagen. Moreover, the protein, containing hydroxyproline and hydroxylysine, has been purified by Kefalides (32) and has been shown, under certain conditions, to precipitate as fibers having the distinct ultrastructure of collagen. These investigators concluded that canine glomerular basement membrane is composed of a collagen-like protein as well as a glycoprotein. The data presented in this paper, showing basement membrane disorganization after collagenase treatment, are in good agreement with the view that a collagen-like protein contributes to the macromolecular aggregate morphologically visualized as the basement membrane of the dermo-epidermal junction.

It is of interest to note that at the dermo-epidermal junction a distinctive type of periodic filament, inserting into the basement membrane, has been described by Palade and Farquhar (33). Perhaps this is the aggregated form of the special basement membrane-type of collagen.

The tendency for fragments of damaged basement membrane to adhere to the basal cell border at the sites of hemidesmosomes in collagenase blisters is, perhaps, indicative of an increased attachment affinity between basement membrane and basal cell plasma membrane at these sites. The structural correlate of this may be represented by the fine filaments linking the basement membrane with the hemidesmosome.

The collagen fibrils in the upper dermis did not demonstrate any ultrastructural alterations except after relatively prolonged exposure (ten minutes) to high concentrations of the enzyme (2.0%). Thus, in the collagenase blisters the basement membrane apparently was more susceptible to the action of the enzyme and the collagen fibrils of the dermis were somehow spared from enzymatic damage. One explanation of this may be, as proposed by Mathews (34), that the collagen fibrils possess a glycoprotein coat. Such a coat could hinder the access of collagenase to the collagen "core" and prevent the rapid degradation of collagen by the enzyme in vivo.

Blisters produced by long exposures to relatively high concentrations of collagenase had features of both basement membrane dis-

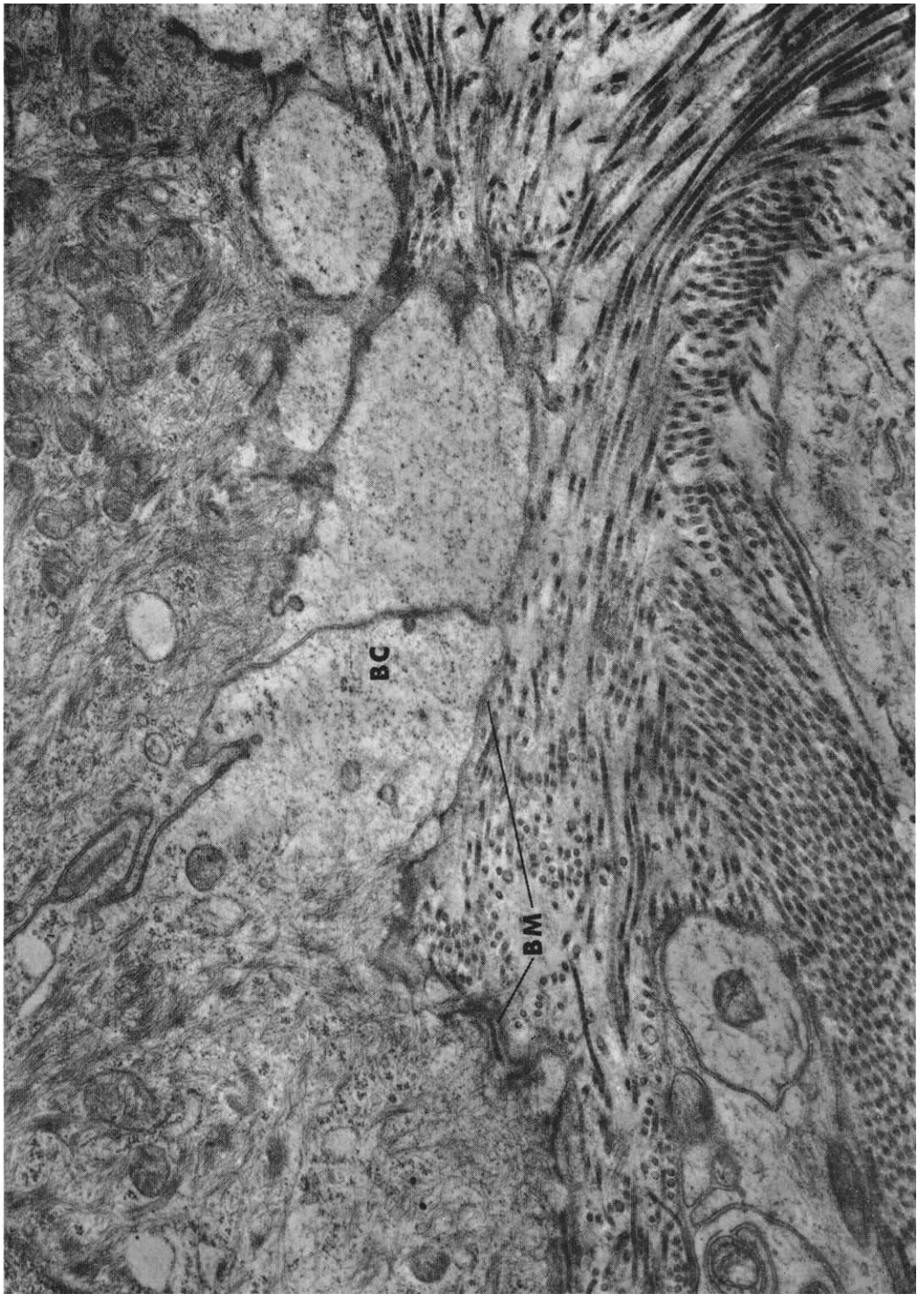

FIG. 12. 0.2% highly purified collagenase, six minute biopsy. Dermal-epidermal junction away from main blister. Pale extensions of basal cell cytoplasm (BC) are present and are covered by basement membrane (BM) which is relatively indistinct, especially on the right side of the micrograph. Approximately 13,500 ×.

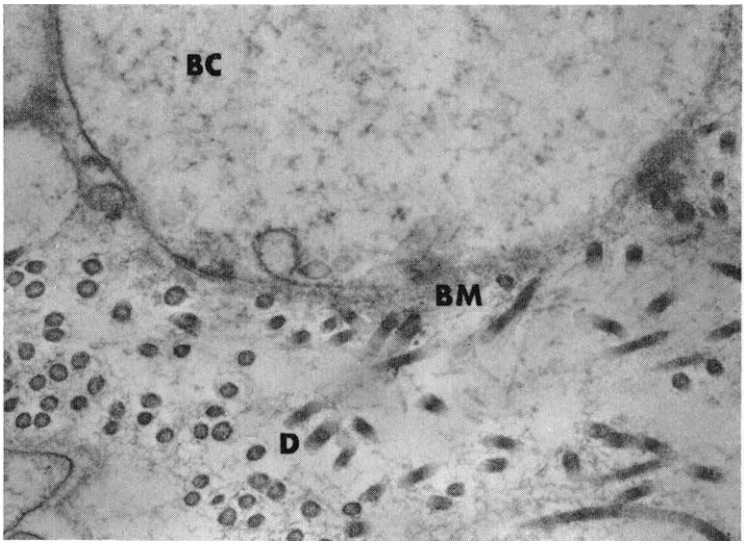

FIG. 13. 0.04% highly purified collagenase, twenty minute biopsy. Dermal-epidermal junction away from main blister. A highly rarefied pale extension of basal cell cytoplasm (BC) is covered with a relatively thin and ragged appearing basement membrane (BM). The dermis (D) is edematous. Approximately 38,000 ×.

organization and degeneration of the collagen fibrils of the upper dermis. Such blisters morphologically are similar to those seen in epidermolysis bullosa dystrophica (4, 6).

Basal cell damage observed in the collagenase blisters consisted chiefly of extrusions of membranous processes into the blister cavity most often at sites between hemidesmosomes and where defects in the basement membrane were noted. This suggests that there is an increased pliability of the basal cell plasma membrane between hemidesmosomes. Other changes observed in the basal cell such as perinuclear vacuolization and mitochondrial swelling are indicative of a nonspecific cellular response to injury.

As in the papain induced blisters (12) free melanocytes were noted to be present in the cavities of blisters produced by collagenase. This may represent a general response of melanocytes to dermal-epidermal separation and reflect the lack of an organized intracellular attachment apparatus between melanocytes and epidermal cells. The occasional free nuclei observed in the blister cavities were derived from intact melanocytes and may indicate that melanocytes are more fragile than basal cells after exposure to noxious stimuli.

SUMMARY

The effects of intracutaneously injected Clostridial collagenase on guinea pig skin was studied by both light and electron microscopy. Clean dermal-epidermal separation occurred rapidly after injection of the enzyme and ultrastructural studies revealed consistent disorganization of the basement membrane in such blisters. The collagen fibrils of the dermis were structurally unaltered by collagenase except after prolonged exposure to relatively high concentrations of the enzyme when collagen damage was observed. Damage to the basement membrane induced by collagenase suggests that the basement membrane contains, as part of its structure, a collagen-like protein which apparently is more sensitive to the degradative effect of Clostridial collagenase than are the collagen fibrils of the dermis.

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